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A Small C-Terminal Sequence of Aurora B Is Responsible for Localization and Function

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Aurora B, a protein kinase required in mitosis, localizes to inner centromeres at metaphase and the spindle midzone in anaphase and is required for proper chromosome segregation and cytokinesis. Aurora A, a paralogue of Aurora B, localizes instead to centrosomes and spindle microtubules. Except for distinct N termini, Aurora B and Aurora A have highly similar sequences. We have combined small interfering RNA (siRNA) ablation of Aurora B with overexpression of truncation mutants to investigate the role of Aurora B sequence in its function. Reintroduction of Aurora B during siRNA treatment restored its localization and function. This permitted a restoration of function test to determine the sequence requirements for Aurora B targeting and function. Using this rescue protocol, neither N-terminal truncation of Aurora B unique sequence nor substitution with Aurora A N-terminal sequence affected Aurora B localization or function. Truncation of unique Aurora B C-terminal sequence from terminal residue 344 to residue 333 was without effect, but truncation to 326 abolished localization and function. Deletion of residues 326–333 completely abolished localization and blocked cells at prometaphase, establishing this sequence as critical to Aurora B function. Our findings thus establish a small sequence as essential for the distinct localization and function of Aurora B.

INTRODUCTION

Cell division requires the precise coordination of a number of events. The passenger proteins, described for the first time more than a decade ago (Earnshaw and Bernat, 1991), are involved in two such events: chromosome segregation and cytokinesis. However, the role of these proteins is still poorly understood (for review, see Adams *et al*., 2001a). All passenger proteins exhibit a specific distribution pattern during mitosis. At metaphase, they associate with inner centromeres and upon the transition to anaphase, they translocate to the spindle midzone and finally localize to the midbody during cell cleavage. To date, several different passenger proteins have been identified: INCENP (Cooke *et al*., 1987), survivin (Skoufias *et al*., 2000), TD60 (Andreassen *et al*., 1991; Martineau-Thuillier *et al*., 1998), ORC6 (Prasanth *et al*., 2002), Borealin/Dasra B (Gassmann *et al*., 2004; Sampath *et al*., 2004), and Aurora B (Adams *et al*., 2000). In both *Caenorhabditis elegans* and *Drosophila*, depletion by RNA interference (RNAi) of one of the passenger proteins, either INCENP, survivin, or Aurora B, results in mislocalization and redistribution of the other passenger proteins (Kaitna *et al*., 2000; Speliotes *et al*., 2000; Adams *et al*., 2001b). Similar results have been obtained in mammalian cells (Mollinari *et al*., 2003). Biochemical studies have demonstrated that INCENP, survivin, Dasra B, and Aurora B exist as a complex in extracts isolated from *Xenopus* eggs (Adams *et al*., 2000; Bolton *et al*., 2002). In vitro experiments have shown that survivin interacts directly with both INCENP and Aurora B (Adams *et al*., 2000; Wheatley *et al*., 2001).

Aurora A, a paralogue of Aurora B, is a mitotic serine-threonine kinase that exhibits localization and activity that are distinct from Aurora B (Bischoff and Plowman, 1999; Giet and Prigent, 1999). Whereas Aurora B is a passenger protein with a role in chromosome segregation and cell cleavage, Aurora A has a role in centrosome function (Nigg, 2002). The two proteins seem to have derived from a single progenitor, Ipl1, present in yeast (Bischoff and Plowman, 1999). Genetic analysis of the single Aurora kinase Ipl1 in *Saccharomyces cerevisiae* has suggested that Ipl1 is involved in chromosome segregation (Chan and Botstein, 1993; Biggins *et al*., 1999), and recent reports offer evidence that Ipl1 is required to monitor tension at kinetochores (Biggins and Murray, 2001; Stern and Murray, 2001; Tanaka *et al*., 2002).

Although the central catalytic domains of Aurora A and Aurora B share substantial homology, the N terminus of the two proteins is highly divergent, and it would be reasonable to assume that the divergent sequences relate to the highly divergent functions of the two proteins.

The difference of function between the two proteins has been made evident by RNAi ablation. In *C. elegans* embryos,

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Abbreviations used: HA, hemagglutinin; siRNA, small interfering RNA.

Aurora B is involved in chromosome segregation, midbody organization, polar body extrusion, completion of cytokinesis, and histone H3 phosphorylation (Schumacher *et al*., 1998; Speliotes *et al*., 2000). In *Drosophila*, Aurora B plays multiple roles in cell division, being involved in metaphase chromosome alignment, kinetochore disjunction, chromosome segregation, and histone H3 phosphorylation (Adams *et al*., 2001b; Giet and Glover, 2001).

In contrast, Aurora A, does not localize to inner centromeres or the cleavage furrow, but associates with the microtubule binding protein TPX2 (Kufer *et al*., 2002) and is associated with the centrosome and mitotic spindle during mitosis (Bischoff *et al*., 1998; Zhou *et al*., 1998; Kufer *et al*., 2002). Aurora A is required for centrosome maturation and formation of a bipolar mitotic spindle (Hannak *et al*., 2001), for mitotic checkpoint function (Anand *et al*., 2003), and for accurate segregation of both centrosomes and chromosomes into daughter cells during mitotic exit (Bischoff and Plowman, 1999; Dutertre *et al*., 2002; Meraldi *et al*., 2002).

Despite the high interest in their functions, there is little information on the sequence requirements for the distinct targeting and functions of Auroras A and B. In this work, we have used a combination of small interfering RNA (siRNA) (Elbashir *et al*., 2001; Brummelkamp *et al*., 2002) depletion of Aurora B and restoration of function with Aurora B truncation mutants to determine the domain requirements for Aurora B function in mammalian cells in culture. We here report that a short C-terminal sequence of Aurora B is required for localization to the inner centromeres at metaphase and for Aurora B function.

MATERIALS AND METHODS

Plasmid Constructions

pcDNA-HA Aurora-B and pcDNA-HA Amino-Terminal Deletion Aurora Constructs. The full-length Aurora B sequence was polymerase chain reaction (PCR)-amplified from human fetal liver Marathon-ready cDNA by using the forward AurB(+) 5′-cgcggatccgcccagaaggagaactcctac-3′ and the reverse AurB(−) 5′-ccgccgctcgagggccgacagattgaagggc-3′ oligonucleotides. Amino-terminal deletions of Aurora B were constructed by PCR amplification by using the following three forward oligonucleotides: 5′-cgcggatccggcctgagcacctgccccag-3′, 5′-cgcggatccgctgcccctggccagaaggtg-3′, and 5′-cgcggatccgacatcttaacgcggcacttcac-3′, and AurB(−) reverse oligonucleotide. The hemaglutinin (HA)-Aurora B constructs were built by cloning the restricted fragments *Bam*HI-*Xho*I containing the full-length coding sequence of Aurora B or the amino-terminal deleted coding sequence of Aurora B into the HA-pCDNA3(+) (Invitrogen, Carlsbad, CA). The resulting HA tag resides at the NH_2-terminal of the Aurora B constructs.

siRNA Nondegradable Aurora B. Seven mutations were introduced in the region of Aurora B HA-tagged construct that was complementary to the siRNA by site-directed mutagenesis by using the following primers: (AurB7(+), 5′-gtcctccggaaGgaAccAgtAacTccTtcGgcacttgtc-3′ and AurB7(−), 5′-gacaagtgcCgaAggAgtTacTggTtcCttccggaggac-3′. The substituted nucleotides are indicated with capital letters.

siRNA Nondegradable Aurora B with Deletion of Amino Acids 7–11 (Aurora-B*). Amino acids 7–11 of the HA-tagged siRNA nondegradable clone of Aurora B were deleted by site-directed mutagenesis by using the primers AurBΔ7-11+ (5′-gatccgcccagaaggagaactacggccgacagacggctcc-3′) and AurBΔ7-11− (5′-ggagccgtctgtcggccgtagttctccttctgggcggatc-3′).

pcDNA-HA Dead Kinase Aurora-B*. The kinase inactive mutant of Aurora B, pcDNA-HA Dead Kinase Aurora-B*, was made by site-directed mutagenesis of the pcDNA-HA-Aurora-B*described above. The critical lysine (K) at position 106 was substituted by alanine (A) by using the primers AurBK/A+ (5′-gccatttcatcgtggcgctcGCTgtcctcttcaagtcccagatag-3′) and AurBK/A− (5′-ctatctgggacttgaagaggacAGCgagcgccacgatgaaatggc-3′).

pcDNA-HA-Carboxyterminal-Deletion-Aurora-B* Constructs. The three constructs were obtained using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) by using three pairs of oligonucleotides: 5′-g a a c g g c t-g c c c c t g g c c c a g g t c t c a g c c c a c c c t t g g t g a c t c g a g t c-3′/5′-g a c t-c g a g t c a c c a a g g g t g g g c t g a g a c c t g g g c c a g g g g c a g c c g t t c-3′, 5′-c t t g g g t c c g g g c c a a c t c t t g a c t c g a g t c t a g a g g g c c c g-3′/5′-c g g g c c c t c t a g a c t c g a g t c a a g a g t t g g c c c g g a c c c a a g -3′, and 5′-c g g g c c a a c t c t c g g a g g t g a c t c g a g t c t a g a g g g c c c g-3′/5′-c g g g-c c c t c t a g a c t c g a g t c a c c t c c g a g a g t t g g c c c g-3′ on the pcDNA-HA-Aurora-B* matrix.

The internal deletion HA-Aurora-B Δ326-333 was obtained using QuikChange site-directed mutagenesis kit with the following pair of primers, 5′-ccaggtctcagcccacccttgggtgctgcctccctctgcccttc-3′/5′-gaagggcagagggaggcag-cacccaagggtgggctgagacctgg-3′, on the pcDNA-HA-Aurora-B* matrix.

pcDNA-HA-AuroraA1-133-AuroraB78-345 Construct. A PCR fragment corresponding to the amino terminal part of Aurora A was amplified using the AurA(+) 5′-cgcggatccgaccgatctaaagaaaactg-3′ and the chimeric *AurA*-AurB(−) 5′-ggacgcccaatctcaaagtc*ttccaaagcccactgcctc*-3′ oligonucleotides. A PCR fragment corresponding to the kinase domain of Aurora B was amplified using the chimeric *AurA*-AurB(+) 5′-*gaggcagtgggctttggaa*gacttttgagattgggcgtcc-3′ and the AurB(−) oligonucleotides. An overlapped PCR product was amplified using both PCR fragments and AurA(+) and AurB(−) oligonucleotides and cloned into the *Bam*HI and *Xho*I sites of pcDNA-HA vector.

pcDNA-HA-AuroraA1-133-AuroraB78-326-AuroraA383-403 Construct.*First step: pcDNA-HA-AuroraA1-133-AuroraB78-344 construct.* A PCR fragment corresponding to the N-terminal part of Aurora A was amplified using AurA(+) (5′-cg*cggatcc*gaccgatctaaagaaaactg-3′) and chimeric *AurA*-AurB(−) (5′-ggacgcccaatctcaaagtc*ttccaaagcccactgcctc*-3′) oligonucleotides. A PCR fragment corresponding to the kinase domain and C terminal part of Aurora B was amplified using chimeric *AurA*-AurB(+) (5′-*gaggcagtgggctttggaa*gacttttgagattgggcgtcc-3′) and AurB(−) (5′-ccgccg*ctcgag*gggccgacagattgaagggc-3′) oligonucleotides. An overlapped PCR product was amplified using both PCR fragments and AurA(+) and AurB(−) oligonucleotides and cloned into the *Bam*HI and *Xho*I sites of pcDNA-HA vector.

Second step: pcDNA-HA-AuroraA1-133-AuroraB78-326-AuroraA383-403 construct. A PCR fragment corresponding to the C-terminal part of Aurora A was amplified using chimeric AB*A*(+) (5′-ggtctcagcccacccttgg*atcacagcaaattcatcaaaacc*-3)′ and AurA(−) (5-ccgccg*ctcgag*agactgtttgctagatgattc-3′) oligonucleotides. A PCR fragment corresponding to AuroraA1-133-AuroraB78-326 was amplified using AurA(+) (5′-cg*cggatcc*gaccgatctaaagaaaactg-3′) and chimeric AB*A*(−) (5′-ggttttgatgaatttgctgtgat*ccaaggggtgggctgagacc*-3′) oligonucleotides. An overlapped PCR product was amplified using both PCR fragments and AurA(+) and AurA(−) oligonucleotides and cloned into the *Bam*HI and *Xho*I sites of pcDNA-HA vector. The construct pcDNA-HA—Aurora A 1-333—Aurora B 78-313—Aurora A 370-403 (Figure 8) was obtained with the same strategy.

siRNA Preparation

Single-stranded Aurora B gene-specific sense (GAAAGAGCCUGUCACCCCAUC) and antisense (AUGGGGUGACAGGCUCUUUUCCG) RNA oligomers were synthesized using 2′-*O*-(tri-isopropyl)silyloxymethyl chemistry (Qiagen S.A., France). Double-stranded RNA was obtained following the manufacturer's instructions. We also used the pSUPER mammalian expression vector that directs the synthesis of siRNA-like transcripts for suppression of Aurora B expression. Two 64-mer oligos, one forward (GATCCCCG-AGCCTGTCACCCCATCTGTTCAAGAGACAGATGGGGTGACAGGCTCT-TTTTGGAAA) and one reverse (AGCTTTTCCAAAAAGAGCCTGTCACCC-CATCTGTCTCTTGAACAGATGGGGTGACAGGCTCGGG) were synthesized (Sigma-Aldrich, St. Louis, MO), annealed, phosphorylated, and ligated into the *Bgl*II and *Hind*III restriction sites of digested pSUPER vector (Brummelkamp *et al*., 2002).

Cell Culture, Transfection, and Immunofluorescence Microscopy

HeLa cells were grown on DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and were grown on poly-D-lysine (Sigma-Aldrich)–coated 12-mm-diameter glass coverslips for at least 24 h before transfection. Cells were transfected with 7 μg of the different pcDNA Aurora B constructs (encoding HA fusion proteins) by using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's protocols. Single-stranded sense and antisense strands, used as controls, and siRNA duplexes were introduced into the cells using Oligofectamine (Invitrogen). For double plasmid transfections (pSUPER-mediated siRNA and pcDNA Aurora B constructs), 5 μg of each plasmid was introduced using Lipofectamine. Routinely, cells 48 h after transfection were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at 37°C. Cells before staining were permeabilized with 0.2% Triton X-100 in PBS for 3 min and then incubated with primary and secondary antibodies.

Endogenous Aurora B was detected by monoclonal AIM-1 antibody (BD Transduction Laboratories, Lexington, KY) used at 100-fold dilution, and by a rabbit polyclonal peptide antibody to N-terminal peptide (amino acids 1–17) of human Aurora B (Crosio *et al*., 2002) used at 500-fold dilution; ectopically expressed HA-Aurora B was detected by anti-HA monoclonal antibody (mAb) (Babco, Richmond, CA) used at 1500-fold dilution. JH human autoim-

mune serum (Andreassen *et al*., 1991) and rabbit polyclonal anti-survivin (Novus Biological, Littleton, CO) were used at 500-fold dilution to detect TD-60 and survivin, respectively. Phosphorylated H3 was detected with phospho-specific polyclonal antibodies to serine 10 and to serine 28 (both from Upstate Biotechnology, Lake Placid, NY) at 500-fold dilution each. Monoclonal IAK1 antibody (BD Transduction Laboratories) recognizing the N terminus of Aurora A was used at 100-fold dilution. Secondary antibodies, including fluorescein isothiocyanate-conjugated affinity-purified goat anti-mouse and anti-rabbit antibodies, and rhodamine-conjugated antihuman antibodies, were used at 250-fold dilution (all from Jackson ImmunoResearch Laboratories, West Grove, PA). An Alexa Fluor 568-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) also was used at 250-fold dilution. DNA was detected by incubation with 0.2 μg/ml propidium iodide in PBS for 5 min after incubations with secondary antibodies. Images were collected with an MRC-600 laser scanning confocal apparatus (Bio-Rad, Hercules, CA) coupled to a Nikon Optiphot microscope.

Cell Extracts and Immunoblotting

Twenty-four hours after transfection, cells were blocked in mitosis for additional 24 h in the presence of 0.1 μg/ml nocodazole (Sigma-Aldrich). Mitotic cells were harvested by mitotic shake off and lysed in SDS 1× sample buffer containing 6 M urea. Lysates were boiled, sonicated, and loaded onto polyacrylamide gels and then transferred to nitrocellulose. Endogenous Aurora B was detected by AIM-1 mAb (BD Transduction Laboratories) and anti-Aurora B N-terminal peptide antibody and ectopically expressed HA-Aurora B with an anti-HA mAb (Babco). Nitrocellulose sheets were then incubated with horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG secondary antibodies. Protein-antibody complex was detected by enhanced chemiluminescence (Pierce Chemical, Rockford, IL).

RESULTS

In agreement with the previous observations, we have found that human Aurora B kinase, expressed as a HA-tagged chimera, exhibits a distribution pattern typical of passenger proteins in HeLa cells (Figure 1A). At metaphase, Aurora B kinase was present on centromeres (Figure 1A) where it colocalized with other passenger proteins (Figure 3). As mitosis proceeded through anaphase, Aurora B dissociated from centromeres and accumulated at the cleavage furrow and the midbody (Bischoff *et al*., 1998; Terada *et al*., 1998; Crosio *et al*., 2002). By contrast, HA-tagged Aurora A localized to the centrosomes and to the mitotic spindle, as observed previously (Gopalan *et al*., 1997; Kimura *et al*., 1997; Bischoff *et al*., 1998; Crosio *et al*., 2002). It is important to note that the localization observed occurs in the context of overexpression of HA-tagged protein. We have observed no difference in localization relative to native protein, nor interference in function with overexpressed protein.

The distinct localization and functions of Aurora A and Aurora B must be dictated by differences in sequence. For reference, we show sequence alignment between the two human kinases (Figure 1B), with identical sequences highlighted in yellow. Both the N-terminal and C-terminal regions of the two proteins contain divergent sequences, whereas the central catalytic domain has a high degree of homology. Our purpose has been to determine the sequence within Aurora B that defines its unique localization and function.

As has been observed previously (Ditchfield *et al*., 2003; Hauf *et al*., 2003), treatment with siRNA resulted in a strong decrease in Aurora B content in mitotic cells, as determined by immunofluorescence analysis (Figure 2A) and by Western blots (Figure 2B). At 48 h posttransfection, 46 ± 1% of all mitotic cells were negative for Aurora B (Figure 2C), and suppression persisted to 72 h. Aurora B-depleted cells exhibited several mitotic defects, including metaphase misalignment and missegregation of chromosomes, characterized by lagging chromatids during anaphase (Figure 2A). At 72 h posttransfection, 77 ± 1% of all mitotic cells negative for Aurora B were in prometaphase compared with 18 ± 4% of Aurora B-positive cells. Despite its reported role in histone H3 phosphorylation (Adams *et al*., 2001b; Giet and Glover, 2001), Aurora B depletion had no detectable effect on chromosome condensation: the compactness of mitotic chromosomes in Aurora B-silenced cells seemed very similar to the state of chromosomes in nontransfected cells.

As expected from previous work (Adams *et al*., 2001b; Hauf *et al*., 2003), the ablation of Aurora B resulted in a dramatic increase in polyploidy, as assayed by the presence of binucleate and multinucleate cells. At 72 h posttransfection, 28 ± 4% of the total cell population versus 3% of the untreated cells were multinucleated. In addition, treatment with siRNA affected the prometaphase–metaphase transition, yielding a significant increase of the percentage of mitotic cells at prometaphase in transfected cells in comparison with control nontransfected cells.

Ectopic Replacement of Aurora B and Restoration of Aurora B Function

Absence of localization of Aurora B may indicate a reduced affinity for binding sites rather than an absence of function. In this case, correct localization and function can be restored in the absence of the competing wild-type protein. We have thus coupled depletion of the endogenous Aurora B with siRNA treatment and expression of mutant Aurora B within the same cell (Figure 2D), in a "pseudogenetics" approach. Briefly, taking advantage of the degenerate genetic code, we have made both "wild-type" and truncation mutant Aurora B HA-tagged cDNA constructs that could not be suppressed by siRNA treatment (see *Materials and Methods*). In addition, to separately detect either endogenous or ectopically expressed Aurora B in the same cell, a sequence of five amino acids (7–11) was deleted from the N terminus of each of these HA-tagged constructs. Such mutants that are silent for the Aurora B N-terminal antibody (Crosio *et al*., 2002) are referred to as "Aurora B*." We were thus able to detect the endogenous kinase by using an anti-peptide antibody that specifically recognized the five-amino acid sequence (7–11) of the N terminus of Aurora B, both by Western blot (Figure 2E) and by immunofluorescence (Figure 2F). In contrast, the antibody did not recognize ectopically expressed wild-type kinase (Aurora B*), because these five amino acids were deleted from the sequence (Figure 2, E and G). Instead, the ectopically expressed protein was detected with an anti-HA antibody (Figure 2, E and G). Note that the rhodamine secondary antibody used to detect the N-terminal epitope in Figure 2G recognized a nonspecific antigen at the cell cortex. Despite this nonspecific signal, the N-terminal antibody did not localize to chromosomes or to the midbody. No cortical stain was seen with the same primary antibody visualized with a fluorescein-coupled secondary antibody (Figure 2F).

These results permitted cotransfection with both siRNA and HA-tagged Aurora B* to rescue function. In such cotransfection experiments, the ectopically expressed wild-type Aurora B* localized properly in siRNA-treated cells, whereas no signal was observed by the N terminus antipeptide antibody, demonstrating an efficient ablation of the endogenous Aurora B in the same cells (Figures 2G and 3B).

Importantly, the overexpression of the wild-type Aurora B* in the siRNA-treated cells completely reversed the siRNA phenotype, restoring correct chromosome alignment. Thus, 46% of all mitotic double-transfected cells were aligned in metaphase, whereas 75% of cells transfected with siRNA alone were in prometaphase. Cell cleavage was also normal (Figure 3B), and consequently mononucleate cells were maintained at levels comparable with controls that were not treated with siRNA. Thus, 5 ± 2% of double-transfected cells were multinucleated versus 3 ± 3% of controls at 48 h.

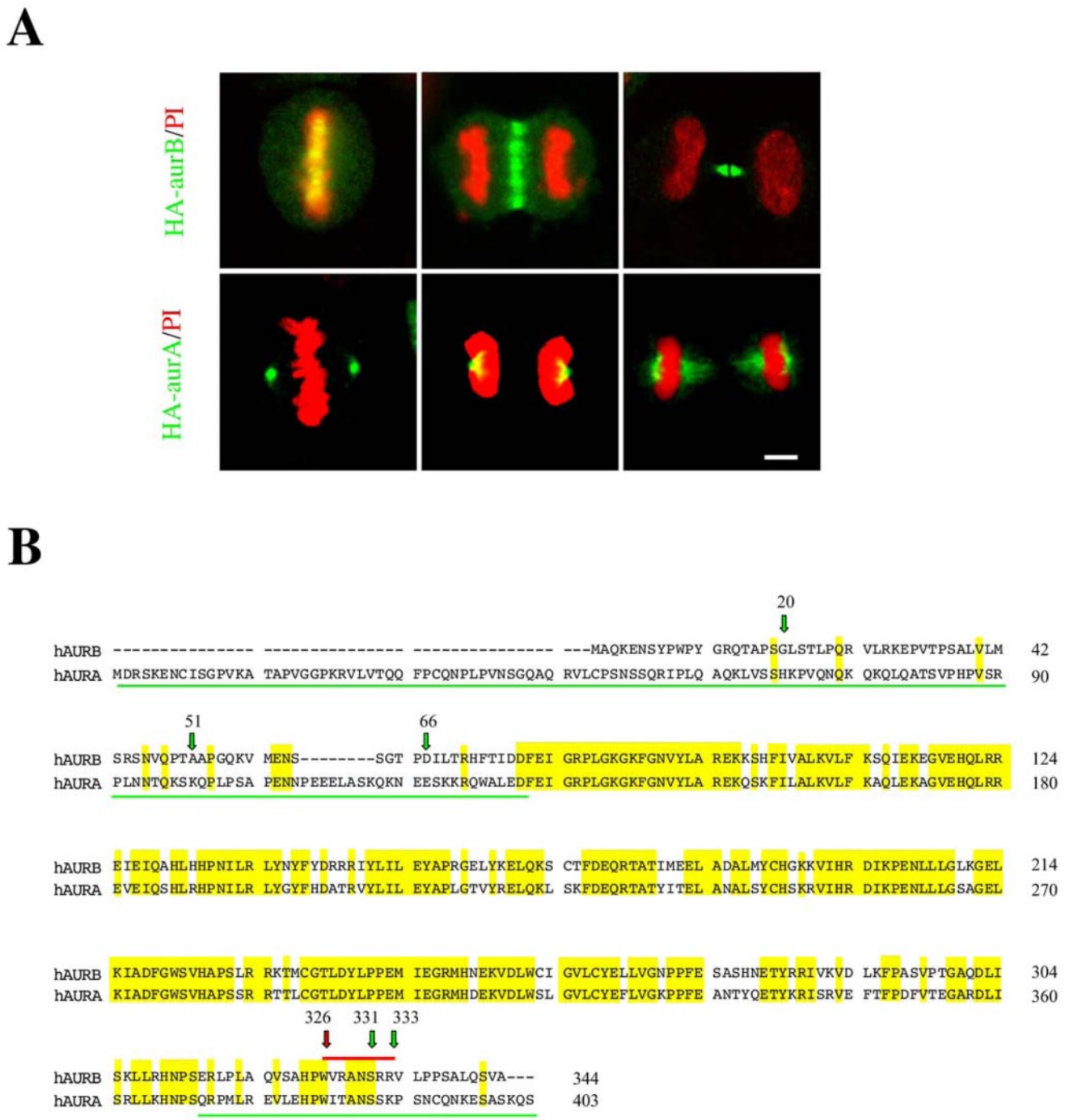

Figure 1. Differences in spindle localization and amino acid primary sequence between Aurora B and Aurora A. (A) Immunofluorescence analysis of metaphase HeLa cells transfected with human HA-Aurora B and HA-Aurora A. Forty-eight hours after transfection cells were stained with anti-HA antibody (green) and with propidium iodide to stain DNA (red). (B) Sequence alignment of human Aurora B and Aurora A. Amino acid identity between the two kinases is marked in yellow. Green arrows and red arrows indicate, respectively, the positions of the different N-terminal and C-terminal truncations of Aurora B that retained or ablated function. Lined sequences indicate the Aurora A N-terminal and C-terminal peptides that were substituted for Aurora B sequence in chimeras (lines below sequence) or Aurora B sequence deletion (lines above sequence). Green indicates Aurora B function, and red indicates absence of function. Bar, 5 μm.

Overexpression of Aurora B* (Figure 2E) was not of consequence to the rescue phenotype. Localization of the rescue protein was correct, and restoration of function was complete, both with respect to induction of correct localization of other passenger proteins and restoration of Aurora B function both at metaphase and during cell cleavage.

siRNA silencing of Aurora B severely disturbed the distribution pattern of the passenger proteins survivin and

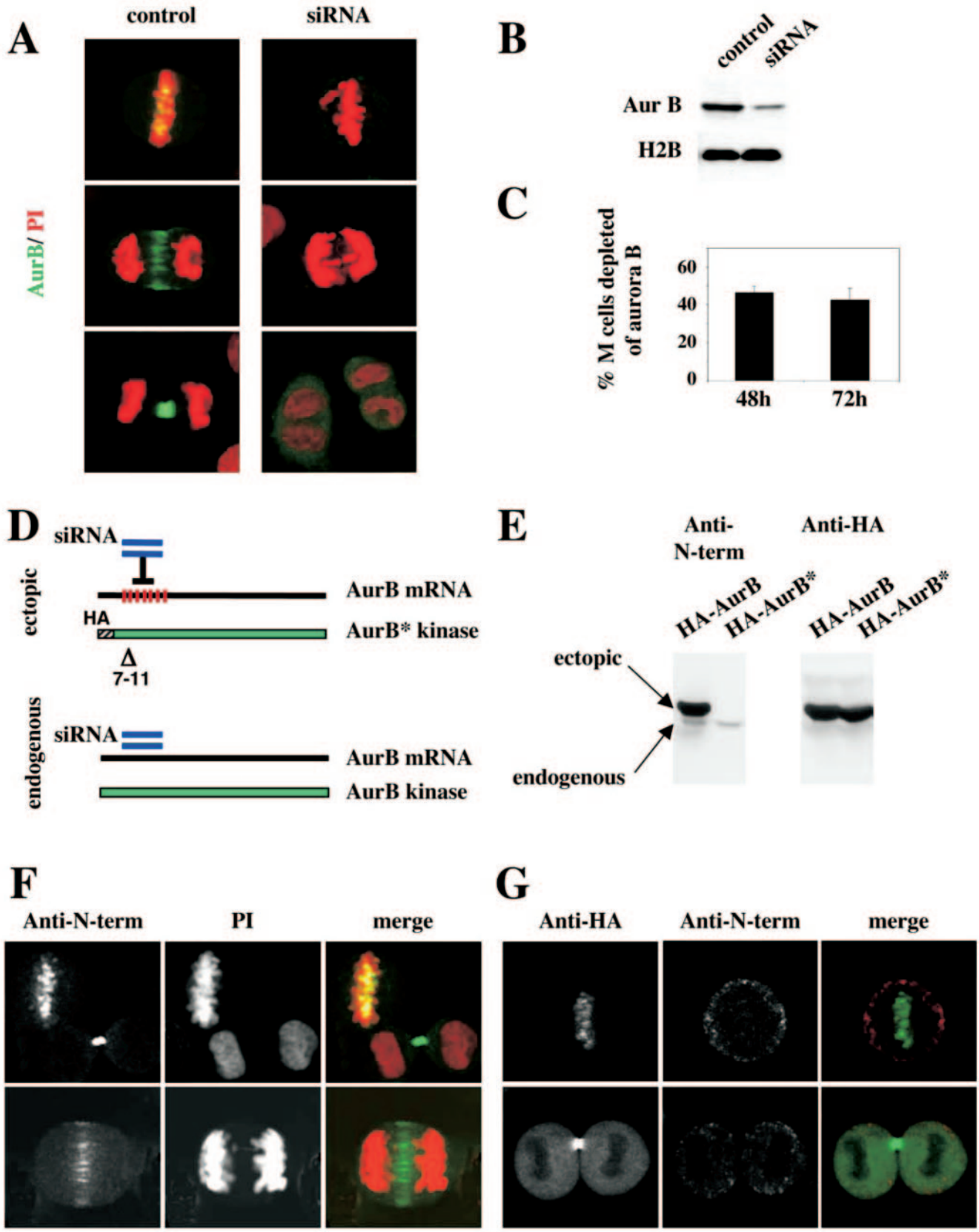

Figure 2. siRNA suppression and simultaneous rescue of Aurora B by ectopic expression. HeLa cells were transfected with siRNA and examined for mitotic and cytokinesis defects. (A) Immunofluorescence microscopy for control and siRNA-treated cells stained with antibody against Aurora B kinase (green) and with propidium iodide to stain DNA (red). siRNA cells exhibit chromosome misalignment at metaphase, aberrant segregation at anaphase, and polyploidy. The cells were observed 48 h after treatment with siRNA commenced. (B) Western blot

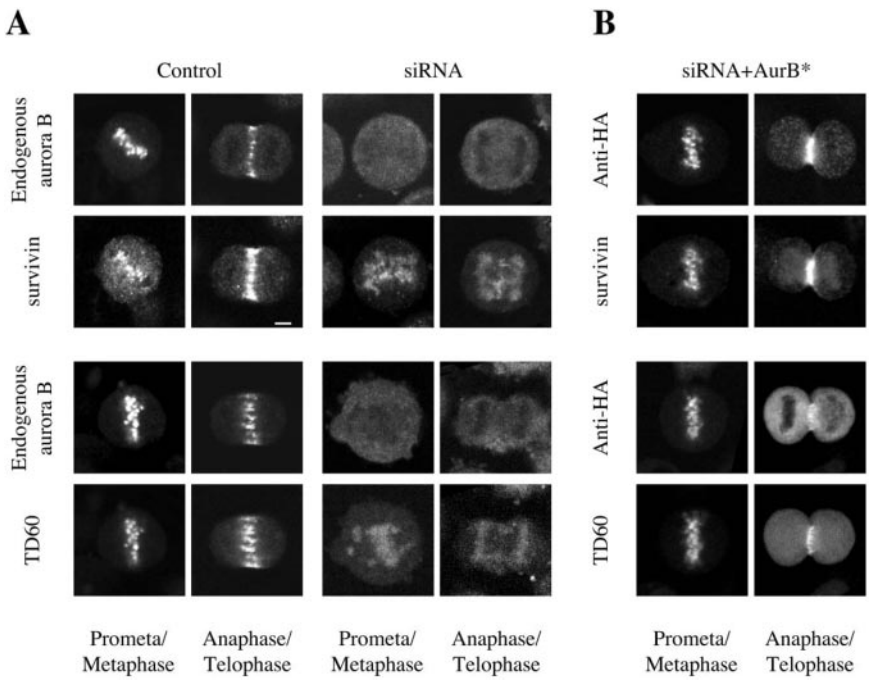

Figure 3. Aurora B rescue also restores normal passenger protein distribution. (A) Depletion of Aurora B leads to redistribution of the passenger proteins. siRNA Aurora B-transfected HeLa cells were stained with anti-Aurora B or anti-survivin antibody, or with human JH autoimmune serum, recognizing TD-60, 48 h after transfection. The passenger proteins are associated with whole chromosomes at metaphase, and they maintain this association into late mitosis in the absence of Aurora B. Comparable images of untransfected controls are shown for the same mitotic stages. (B) Rescue of chromosome passenger protein kinetochore localization in Aurora B siRNA-treated HeLa cells cotransfected with HA-Aurora B*. Ectopically expressed HA-Aurora B* colocalizes to kinetochores with survivin and TD-60, as in controls. In all figure parts, the upper and lower images are recordings of the same cell in two channels. Bar, 5 μm.

Figure 2. (continued). detection of Aurora B and histone H2B in control and siRNA-treated cells at 48 h posttransfection. Note the decrease of Aurora B in the siRNA-treated cells. (C) Histograms showing the percentage of Aurora B-depleted mitotic cells at 48 and 72 h posttransfection with siRNA, as determined by absence of Aurora B immunofluorescence. (D) Schematic presentation of the "pseudogenetic" approach for Aurora B rescue. Taking advantage of the degenerate genetic code, seven point mutations (indicated by red bars) were introduced by site-directed mutagenesis, in the region complementary to the siRNA, in an HA-tagged Aurora B construct without affecting the Aurora B amino acid sequence. Then, a sequence of 15 nucleotides of this new construct, corresponding to the amino acids 7–11 of the N terminus of Aurora B, was deleted (indicated by Δ7-11). This created a form of Aurora B (Aurora B*) that could not be degraded by siRNA, and whose expression could only be detected by an anti-HA antibody, but not by the antibody to the N terminus of the endogenous protein. Endogenous Aurora B was visualized by an anti-peptide antibody raised against the amino acid sequence 1–17 of the N terminus of Aurora B. Amino acid sequence 7–11 of the N terminus of Aurora B was crucial for the recognition of the kinase by the antibody. The double blue bar indicates siRNA, which either can or cannot interact with the message, as indicated. (E) The antibody raised against the amino acid sequence 1–17 of the Aurora B N terminus (anti-N-term) recognizes only Aurora-B kinase with an intact N terminus. Because Aurora B* lacks the 7–11 amino acid sequence, this form of the kinase could not be detected by the anti-N-terminus antibody. HA-Aurora B and HA-Aurora B* were ectopically expressed in HeLa cells and detected by Western blot by using the anti-N-terminus (anti-N-term) and anti-HA antibodies. Arrows indicate the ectopically expressed and the endogenous kinases. Lanes are labeled to indicate Aurora B or Aurora B* expression. (F) Immunofluorescence microscopy detection of the distribution of endogenous Aurora B in HeLa cells by using the anti-N-terminus antibody; PI, propidium iodide. (G) Rescue of normal chromosome alignment and cytokinesis in Aurora B siRNA-treated HeLa cells cotransfected with HA-Aurora B*. Note the complete absence of endogenous Aurora B kinase as seen by the absence of staining by the anti-N-terminus antibody (anti-N-term) at Aurora B sites stained with HA-antibody (red in merge). The rhodamine secondary antibody used with anti N-term primary gave a nonspecific cortical stain. Bar, 5 μm.

TD-60. No concentrated centromeric localization was observed for either antigen in Aurora B-depleted cells. Instead, these antigens were redistributed throughout the mitotic chromosomes in Aurora B-depleted cells (Figure 3A). Additionally, survivin and TD-60 were perturbed in distribution during anaphase, and did not quantitatively transfer to the central spindle as in control cells but remained largely associated with the chromosomes (Figure 3A). These results are in agreement with the available data for the redistribution of the passenger proteins survivin and INCENP in other systems after depletion of Aurora B (Kim *et al*., 1999; Kaitna *et al*., 2000; Speliotes *et al*., 2000; Adams *et al*., 2001a; Tanaka *et al*., 2002; Carvalho *et al*., 2003) and suggest that mammalian Aurora B could be a required structural component of a passenger protein complex.

In contrast to siRNA-transfected cells, Aurora B, survivin, and TD-60 all localized correctly both to centromeres at metaphase and to the spindle midzone in rescued cells (Figure 3B). Thus, in keeping with a rescue phenotype, the distribution of the passenger proteins in rescued cells was indistinguishable from that of the control cells (Figure 3B). Therefore, we conclude that the expressed exogenous kinase Aurora B* can functionally replace the endogenous Aurora B. These results have validated our approach and have permitted us to study the effect of the kinase mutants in a background where endogenous Aurora B is absent.

Expression of Aurora B N-Terminal Truncation Mutants and Their Effects on Mitosis in the Absence of Endogenous Protein

Having established that ectopically expressed Aurora B could functionally replace endogenous Aurora B that had been suppressed by siRNA transfection, we were able to conduct experiments in which wild-type Aurora B would be replaced by truncation mutants. The N-terminal (1–66) residues of Aurora B are highly divergent from the termini of Aurora A (Figure 1), which has a mitotic localization and function that is distinct from Aurora B. We therefore con-

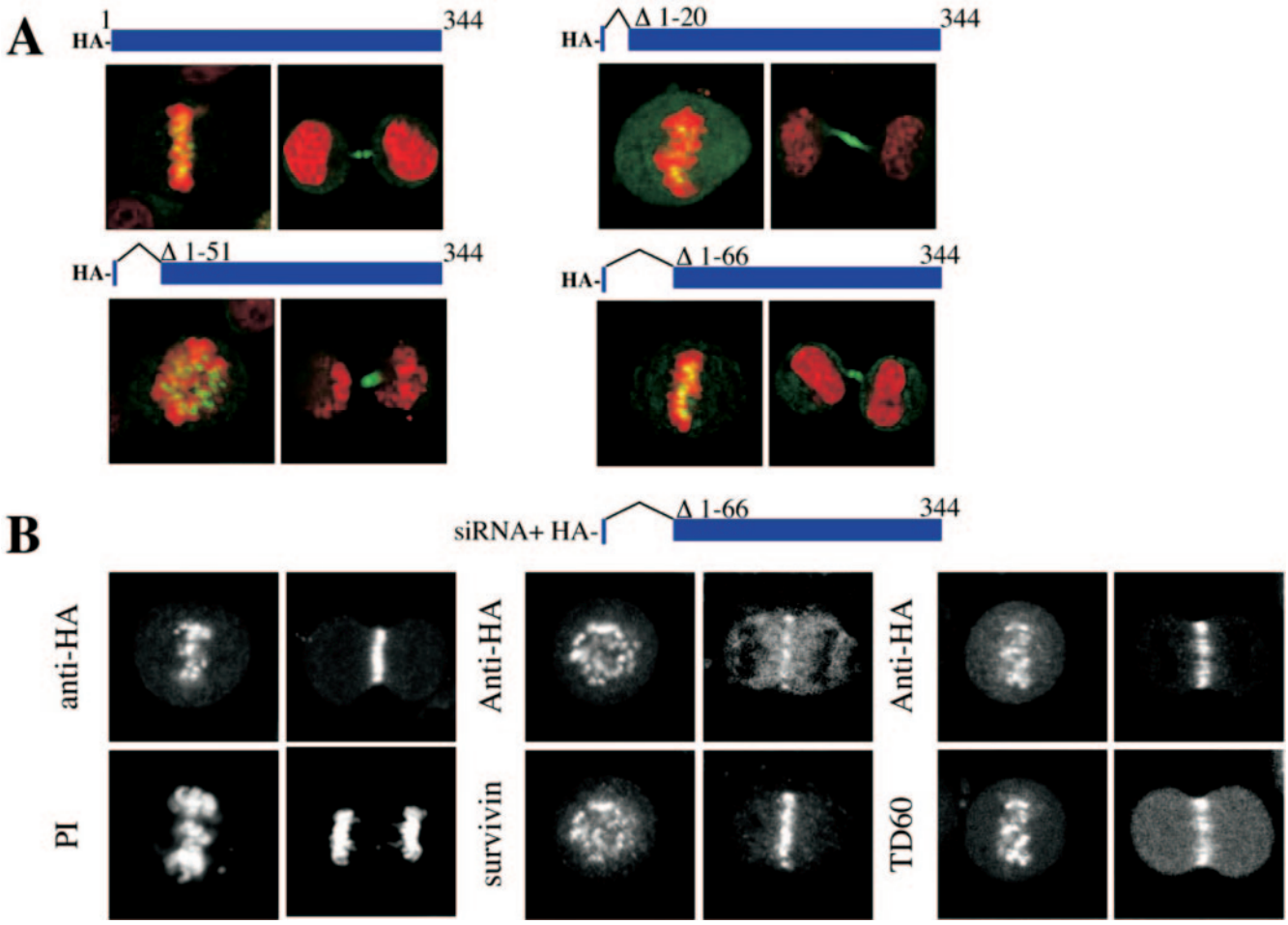

Figure 4. Intracellular localization of N-terminal deletions of Aurora B. (A) Various constructs of N-terminal truncated human Aurora B carrying an N-terminal HA-tag were transfected into HeLa cells. Cells were prepared for immunofluorescence microscopy 48 h posttransfection and stained with anti-HA (green) and with propidium iodide (red). Two mitotic stages for each construct are shown (prometaphase/metaphase and anaphase/telophase). A scheme of the different constructs, with the terminal residues of each construct depicted, is shown on top of the images. The positions of the truncations in the primary sequence are shown by arrows in Figure 1B. (B) Restoration of function in Aurora B siRNA-treated cells, rescued by expression of the longest N-terminal truncation (Δ 1-66), was similarly assayed.

structed a series of HA-tagged N-terminal truncation mutants of Aurora B to conduct functional tests.

First, we determined the effect of overexpression of the different truncation mutants in mitotic cells alone, without suppression of the endogenous protein. Expression of these different truncation mutants in HeLa cells that retained native Aurora B demonstrated that N-terminal truncation mutants of Aurora B were neither displaced from centromeres at prometaphase/metaphase nor from the spindle midzone during anaphase (Figure 4A). Furthermore, none of these mutants had a dominant negative effect on karyokinesis or cytokinesis in the presence of native Aurora B. We then determined the effect of expression of the truncation mutants in the absence of endogenous wild-type Aurora B. Controls in this experimental series showed, as in previous figures, that Aurora B was absent from centromeres in siRNA-transfected mitotic cells and that survivin and TD-60 were both dispersed from metaphase chromosomes (data not shown).

Replacement of native Aurora B by the N-terminal truncation mutant, Aurora B*ΔN 1–66, was without consequence for localization of Aurora B to centromeres or to the spindle midzone and did not interfere with normal completion of cell cleavage (Figure 4B). Furthermore, both survivin and TD-60 localized correctly to the centromeres at metaphase and to the spindle midzone during cell cleavage in Aurora B*ΔN 1–66–expressing cells.

Substitution of the N-Terminal Region of Aurora B by Aurora A Sequence Does Not Alter Aurora B Localization or Function

The lack of effect of the absence of the N terminus of Aurora B on its localization or function permitted us to address whether the divergence of sequence in the N terminus between Aurora A and B derived from a specific N-terminal sequence requirement for correct localization of Aurora A. We tested this possibility by replacing the Aurora B N terminus with the N terminus of Aurora A, creating an HA-tagged chimera, Aurora A 1-133-Aurora B 78-344 (Figure 5A).

The chimeric protein clearly localized to centromeres at metaphase and to the spindle midzone during cell cleavage (Figure 5A) and did not interfere with the proper completion of cleavage. Importantly, the chimera did not localize to centrosomes. Antibody to the N terminus of Aurora A con-

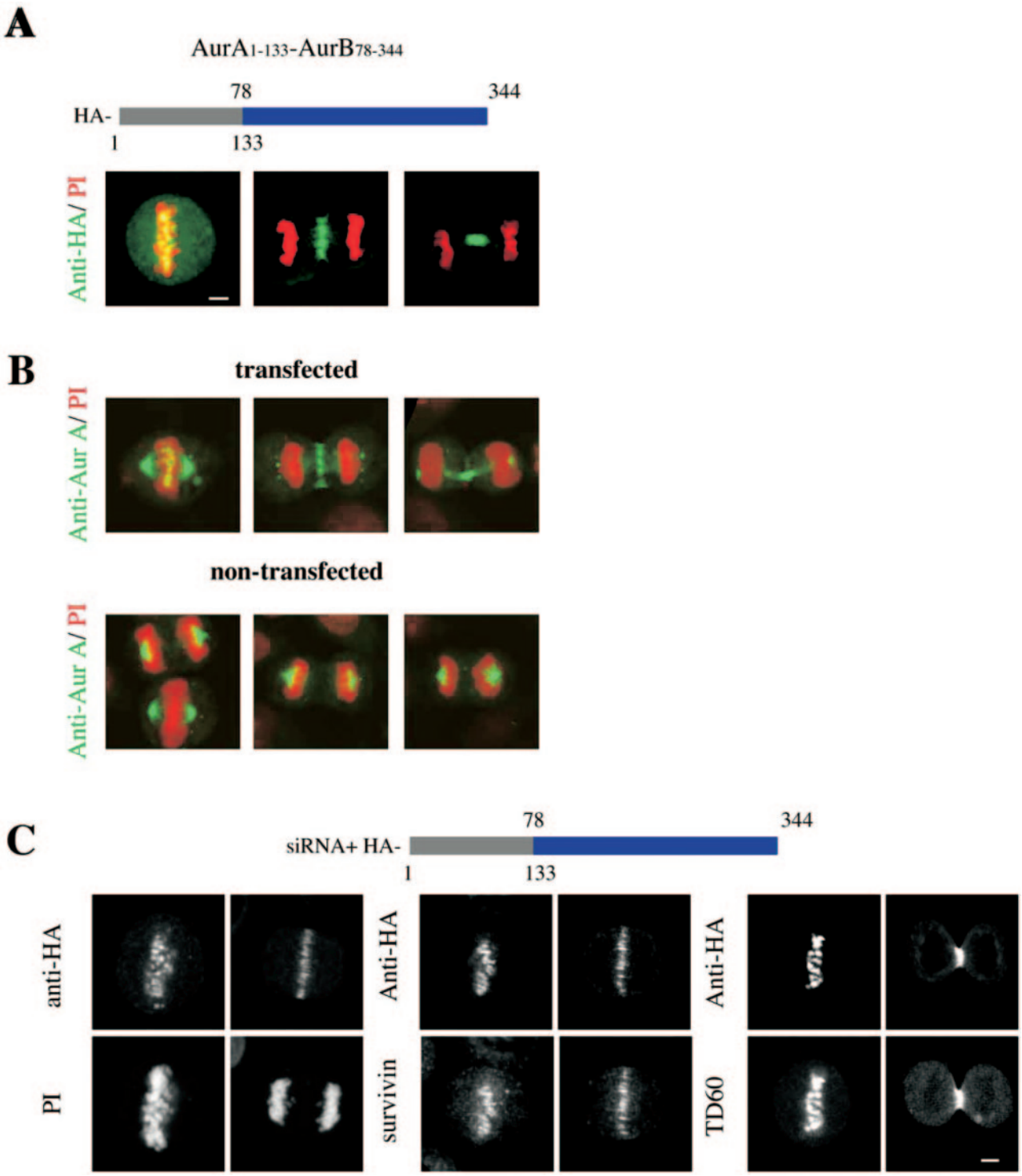

Figure 5. Localization and function of an N-terminal Aurora A-Aurora B chimera alone and in rescue of Aurora B siRNA transfected cells. The schemes of the constructs are shown on top of the images. Numbers above and below the schemes correspond to terminal Aurora B and Aurora A residues, respectively. The Aurora B domain is blue, whereas Aurora A domain swaps are shown in gray. (A) Kinetochore and spindle midzone localization of the HA-Aurora B chimera containing the N-terminal residues (1–133) of Aurora A. Forty-eight hours after transfection cells were stained with anti-HA and with propidium iodide (PI). (B) Transfected (top row) and nontransfected cells (bottom row) stained with a mAb specific for the N terminus of Aurora A and with PI. Note that the anti-AurA recognizes the endogenous protein on the spindle poles and microtubules and the chimeric protein in metaphase at the kinetochores, in anaphase at the spindle midzone and the midbody in cytokinesis. (C) HeLa cells contransfected with siRNA for Aurora B and with plasmid expressing the AurA 1-133-AurB 78-344 chimera (bottom) were stained with anti-HA, with anti-survivin, or with human JH autoimmune serum, recognizing TD-60, and counterstained with PI. Two mitotic stages are shown (prometaphase/metaphase and telophase/cytokinesis, left and right images, respectively). The top and bottom images are recordings of the same cell in two channels. Bar, 5 μm.

firmed that N-terminal Aurora A sequence was present both at centrosomes and at centromeres at metaphase and at centrosomes and the spindle midzone during cell cleavage (Figure 5B). This result demonstrated that the chimera did not interfere with normal Aurora A association with centrosomes and that the chimeric protein specifically failed to localize to centrosomes.

The correct localization of Aurora A 1-133-Aurora B 78-344 to centromeres and to the spindle midzone, and the apparently normal cleavage observed when Aurora B was replaced by this mutant, suggested that the N terminus of Aurora A did not interfere with correct localization of passenger proteins to the centromere or to the spindle midzone. We confirmed this possibility by observing the distribution of survivin and of TD-60 in cells cotransfected with siRNA to Aurora B and with cDNA for Aurora A 1-133-Aurora B 78-344. The distribution of the other passenger proteins was normal in all respects (Figure 5C), both at metaphase (left images) and at anaphase/telophase (right images).

A Specific C-Terminal Region of Aurora B, Amino Acids 326-333, Is Required for Localization and Function

The N-terminal sequence divergence between Aurora B and Aurora A is not determinant for the distinct localization of Aurora B. We therefore focused on determining the effect of C-terminal Aurora B truncations or internal deletion on Aurora B localization and function.

After C-terminal truncation of the sequence divergent from Aurora A (Δ333-344), Aurora B correctly localized both to kinetochores and to the anaphase spindle bundle, either with or without ablation of native Aurora B (Figure 6A). In contrast, further deletion of the C terminus (Δ331–344 and Δ326–344) caused failure to localize in the presence of the native protein, and the ectopic Aurora B was completely dispersed in mitotic cells (Figure 6, B and C, left images). Despite its displacement, the C-terminal truncation mutant had no dominant negative effect on chromosome congression or on cytokinesis.

Despite the mislocalization of Aurora B Δ331-344 in the presence of native protein, it correctly localized and substituted for Aurora B after ablation of the native protein (Figure 6B, right), suggesting weaker affinity for binding sites, but retention of function in the absence of the native protein. In the presence of these mutants, the other passenger proteins correctly localized (Figure 6, A and B). In contrast Δ326-344 did not substitute for native Aurora B after siRNA ablation (Figure 6C), and cells could not proceed past prometaphase/metaphase. Furthermore, the passenger proteins survivin and TD-60 were dispersed as well. These results suggested that residues 326–333 were critical for Aurora B function. We therefore tested the function of Aurora B containing an internal Δ326-333 mutation. This mutant was completely defective in localization in the presence of the native protein (Figure 6D, left), and when substituted for ablated native Aurora B, it could not localize or rescue function. No HA-positive cells were observed to progress past metaphase, and the other passenger proteins dispersed throughout the chromosomes. Our results indicate that the C-terminal region is critical for Aurora B localization to centromeres and also indispensable for the localization of other passenger proteins. The C-terminal region also seems essential for progression past metaphase.

All Aurora B C-Terminal Truncation Mutants Exhibit Histone H3 Kinase Activity

We have found that rescue with the Δ326-344 and the Δ326-333 mutants of Aurora B results in failure of chromosome localization and of progression past metaphase. Although this seems to identify an important functional region of Aurora B, we needed to eliminate the possibility that failure of rescue was due instead to disrupted folding of the enzyme and global failure of function. Careful analysis of the putative Aurora B structure, based on Swiss model alignment with comparable sequence in the Aurora A structure, showed that residue 326 should lie outside the core structure of the enzyme, beyond the last alpha helix, and would be unlikely to be important to structural integrity (our unpublished data).

To confirm this impression, we conducted an analysis of the capacity of the Aurora B mutants to properly phosphorylate two residues on histone H3 (S10 and S28), which are Aurora B substrates (Hans and Dimitrov, 2001; Goto *et al*., 2002). Aurora B siRNA-transfected cells were rescued with different Aurora B mutant constructs, as described above, and mitotic cells were assayed 48 h posttransfection for the presence of the phosphoepitopes for S10 (Figure 7A) and S28 (Figure 7B) on histone H3. As a control, in addition to the C-terminal deletion constructs cells were also rescued with an Aurora B active site point mutant (K106A) that totally suppresses kinase activity (Terada *et al*., 1998). The result clearly demonstrated that all C-terminal mutant constructs retained the capacity to phosphorylate both histone H3 phosphoepitopes in contrast to the K106A mutant, which localized correctly to kinetochores but failed to generate histone H3 phosphoepitope signals in transfected cells (Figure 7).

Rescue with Double N- and C-Terminal Chimeras of Aurora A and Aurora B

Sequence comparison between Aurora B and Aurora A indicates that three of seven residues are identical in the critical 326–333 region of Aurora B (Figure 1B). We therefore asked whether the C-terminal sequence of Aurora A could substitute for the C-terminal sequence of Aurora B to restore function. The chimera containing N-terminal Aurora A sequence and C-terminal Aurora B sequence (Aurora A 1-133-Aurora B 78-344), as described in Figure 4B, was further modified by a C-terminal tail swap of Aurora A sequence (370–403) for aligned Aurora B sequence (313–344). This chimera did not localize either to kinetochores during early mitosis nor to the spindle midzone in anaphase (Figure 8). Remarkably, however, the chimera rescued both localization and function in cells where the native Aurora B had been ablated by siRNA (Figure 8, bottom). We chose a sequence for exchange that included divergent Aurora B sequence upstream of residue 326 (see alignment in Figure 1B) to assay for the broadest possible effect of C-terminal exchange. The same experiment also was conducted with a tail swap of Aurora A sequence 383–403 for Aurora B sequence 326–344, with results comparable with the longer swap (data not shown). We conclude that a small stretch of the C-terminal region from residue 326–333 is critical to Aurora B function, and that the C terminus of Aurora A contains sequence with the latent capacity to substitute for Aurora B function in the absence of native Aurora B.

DISCUSSION

We have conducted a molecular genetic analysis of the function of domains within the primary sequence of Aurora B. Our approach combines the suppression of wild-type Aurora B by siRNA with the overexpression of truncation or domain replacement mutants within the same cell. These mutants were engineered so that they would not be sup-

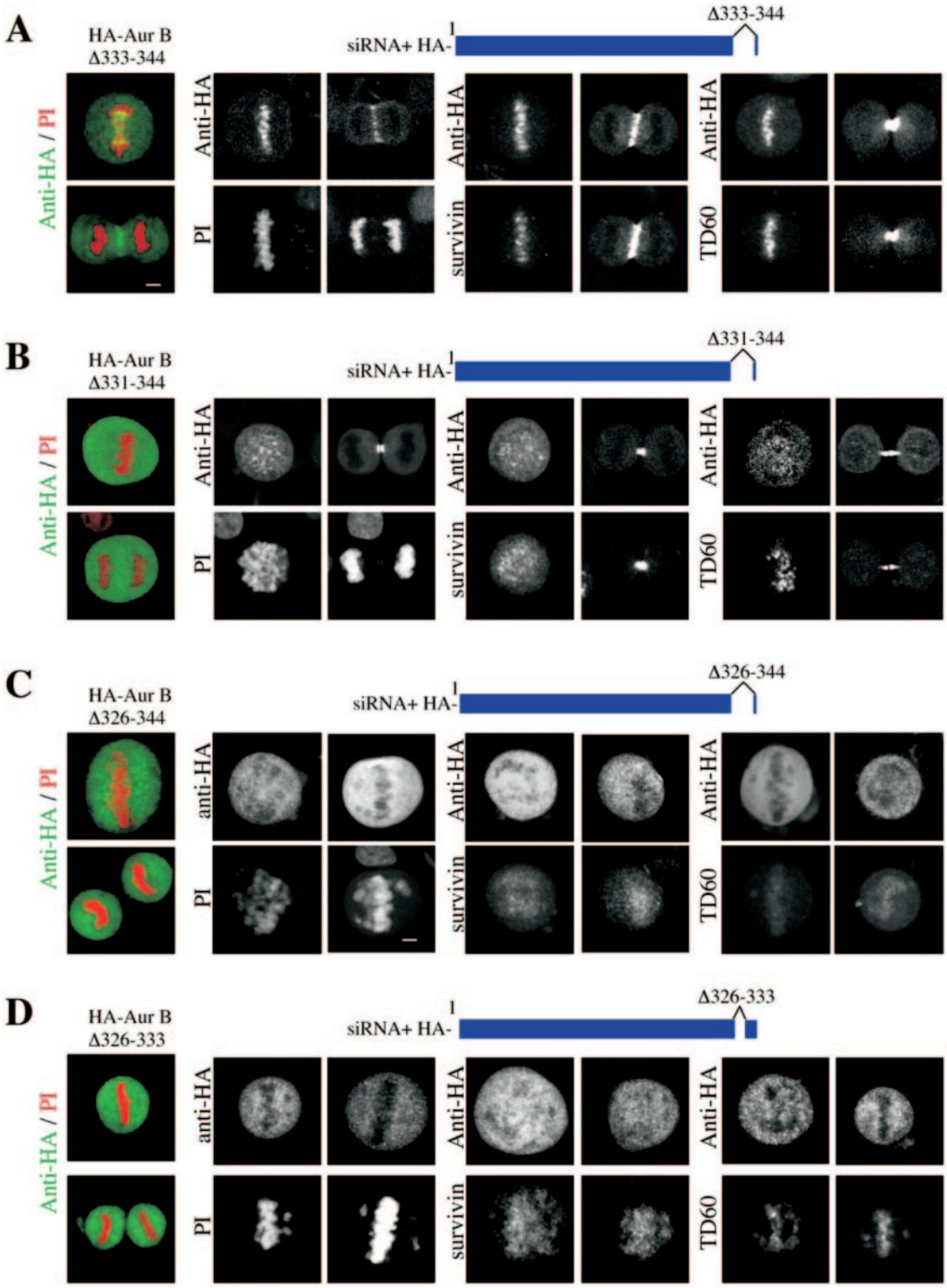

Figure 6. Intracellular localization of C-terminal deletions of Aurora B and restoration of function in siRNA-treated HeLa cells cotransfected with various constructs of Aurora B*. HeLa cells transfected with various constructs were stained with anti-HA, propidium iodide (PI), or anti-survivin antibodies, or with human JH autoimmune serum recognizing TD-60, 48 h after transfection. Two mitotic stages for each construct are shown (prometaphase/metaphase and telophase/cytokinesis), except for rescues shown in C and D, where no mitotic stages after metaphase were found. A scheme of the different constructs, depicting the terminal residues of each deletion, is shown on top of the images. The positions of the truncations in the primary sequence are shown by arrows in Figure 1B. (A and B) Localization and siRNA rescue with HA-Aurora B*1-333 and HA-Aurora B*1-331, respectively. Aurora B*1-331 does not localize in the presence of native protein but rescues function in its absence. C) Localization and siRNA rescue with HA-Aurora B*1-326. This construct does not localize nor rescue function. (D) Localization and siRNA rescue with HA-Aurora B*Δ 326-333. This construct does not localize nor rescue function. Bar, 5 μm.

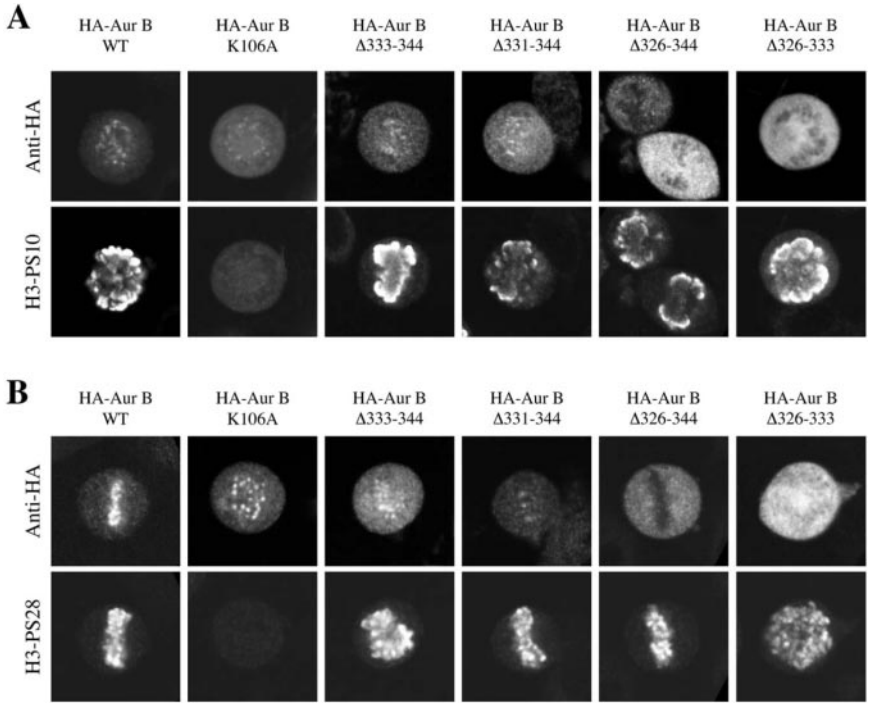

Figure 7. Intracellular kinase activity of the different Aurora B deletions and kinase-dead mutant in the absence of endogenous Aurora B. Cells were cotransfected with siRNA and the various constructs of Aurora B. Cells were than prepared for double immunofluorescence by using anti-HA and H3 phosphospecific antibodies to S10 (A) and S28 (B). Although the HA-AurBK106A mutant localizes to kinetochores, there is almost a complete loss of H3 S10 and H3 S28 phosphorylation. In contrast, H3-S10 and H3-S28 phosphorylation is rescued by the wild-type and all C-terminal deletion mutants of Aurora B irrespective of their ability to associate with the kinetochores. All H3 phosphorylation images were recorded at the same confocal microscope settings.

pressed by siRNA. Furthermore, rescue could be confirmed both by the absence of Aurora B antigen and the presence of an HA-tag. This condition was created by mutation of the cDNA so that the replacement form of Aurora B did not contain the antigenic site recognized by the antibody against wild-type Aurora B. Controls demonstrated that both suppression of endogenous Aurora B, and its rescue by ectopic normal Aurora B, are successful. With this approach, we have demonstrated that the C-terminal region of Aurora B is critical to Aurora B localization and function during cell division. Surprisingly, the homologous region of Aurora A, although divergent, could rescue Aurora B localization and function.

Demonstration of the Important Targeting Role of the C Terminus of Aurora B

The Aurora B catalytic domain is highly homologous in sequence to the catalytic domain of Aurora A, but Aurora B contains an N-terminal domain that diverges substantially from Aurora A sequence. Sequence alignment shows that the divergent N termini of human Aurora A and Aurora B contain 132 residues and 75 residues, respectively. Aurora A and B also have short divergent C-terminal tails, consisting of 16 residues in human Aurora A and 13 in human Aurora B. Aurora B is targeted to centromeres and to the spindle midzone during mitosis, whereas Aurora A associates with the centrosome and with the mitotic spindle. These distinct sites of localization undoubtedly are important elements of the discrete functions of the different Aurora kinases.

Aurora A is required for centrosome maturation and formation of a bipolar mitotic spindle, whereas Aurora B is required for proper chromosome segregation in mitosis and for cell cleavage during cytokinesis. Because the majority of the Aurora kinase sequences are highly homologous, it was reasonable to assume that the unique N-terminal sequences were responsible for discrete targeting and therefore for the unique functions of the two kinases. Our results have surprisingly established that this is not the case.

Prior evidence had suggested that the Aurora A N terminus may have a targeting role in *Xenopus* (Giet and Prigent, 2001). It was therefore a surprise to find that ablation of the N terminus of Aurora B was without consequence for its correct localization to both centromeres and to the spindle midzone during mitosis. Furthermore, when native Aurora B was suppressed by siRNA and replaced by N-terminal truncation mutants, the ectopic protein completely mimicked normal Aurora B localization and fully restored Aurora B mitotic function. We have eliminated an alternative explanation that although the N-terminal–truncated Aurora B cannot localize by itself, it may dimerize with residual native Aurora B and arrive passively at the correct sites. Although it is unlikely that we have eliminated all of native Aurora B from transfected cells, this is not likely the case. First, the native form is not evident at the proper locations in rescued cells, whereas the rescue constructs localize correctly (Figure 2G). Second, if it occurred, dimerization should have permitted phosphorylation of histone H3 when cells were rescued with the Aurora B dead kinase mutant, but this was not the case (Figure 7).

Our results thus demonstrate without equivocation that the N-terminal divergent sequence of Aurora B has no role in targeting or function in mitosis. Furthermore, it is noteworthy that absence of the N terminus of Aurora B had no effect on correct localization of the other passenger proteins. The N terminus of Aurora B therefore may not play a crucial role in the formation of the passenger protein complex during mitosis as has been proposed for *Xenopus* Aurora B (Bolton *et al*., 2002).

Having established that the N terminus of Aurora B was without apparent function, we could test the effect of swapping the N terminus of Aurora A in place of the N terminus of Aurora B. If the N terminus of Aurora A had such an addressing function in mammalian cells, the chimeric protein should have been recruited exclusively to the centrosomes, or to the centrosomes in addition to the centromeres. In fact, we found no evidence for the recruitment of the

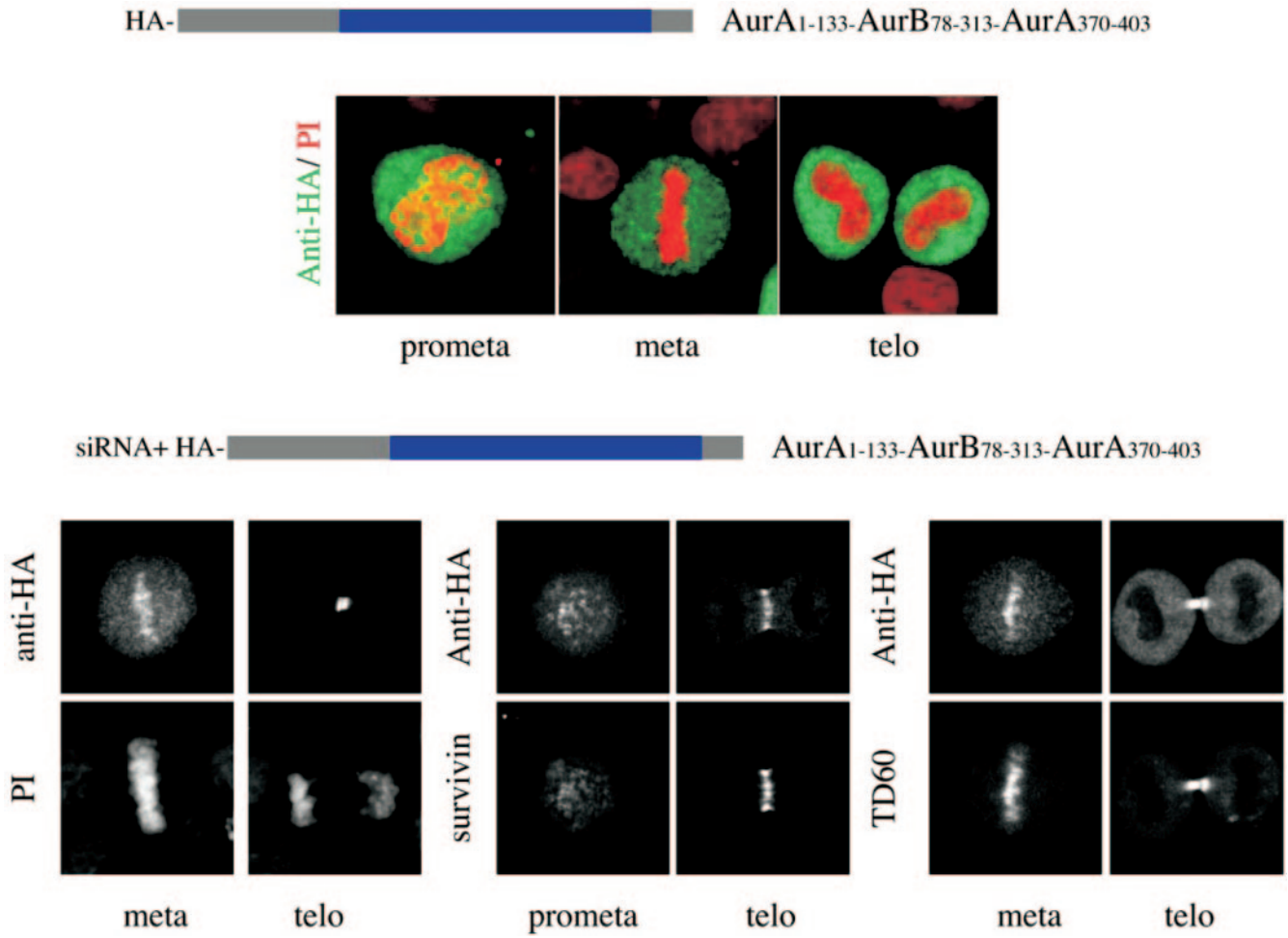

Figure 8. Intracellular localization of an Aurora A-Aurora B chimera with C-terminal Aurora A sequence, and its restoration of Aurora B function in siRNA-transfected cells. Localization and function of the Aurora A-Aurora B (AurA1-133-AurB78-313-AurA370-403) chimera alone, and in rescue of Aurora B siRNA-transfected cells. The scheme of the construct is shown on top of the images. The Aurora B domain is blue, whereas Aurora A domain swaps are shown in gray. Lack of kinetochore and spindle midzone localization of the HA-Aurora B chimera containing N- (1–133) and C-terminal residues (370–403) of Aurora A (top) in prometaphase (prometa), metaphase (meta), or telophase (telo) cells. Forty-eight hours after transfection cells were stained with anti-HA and with propidium iodide (PI). In constrast, HeLa cells contransfected with siRNA for Aurora B and with plasmid expressing the AurA1-133-AurB78-313-AurA370-403 chimera (bottom) showed complete restoration of localization and function, and correctly colocalized with other passenger proteins. Cells were stained with anti-HA, with anti-survivin or with human JH autoimmune serum, recognizing TD-60, and counterstained with PI. The upper and lower images are recordings of the same cell in two channels. Bar, 5 μm.

chimeric protein to centrosomes, and the presence of the N terminus of Aurora A did not influence the localization or function of the ectopic Aurora B chimera in experiments where it replaced the endogenous Aurora B, nor, interestingly, did the chimera alter the targeting of native Aurora A to centrosomes (Figure 5B). It is possible that the targeting efficiency of the Aurora A N terminus is relatively weak and it cannot effectively alter the affinity of the remainder of Aurora B for the centromeres at metaphase or the spindle midzone in anaphase.

Truncation and rescue experiments also have established that the sequence in the C terminus of Aurora B that diverges from Aurora A is similarly without effect on localization. The results are a little more complex than those with the N-terminal truncations and chimeras. Truncation of the C-terminal region to amino acid 333 does not disturb localization or function in the presence or absence of native Aurora B. Interestingly, the loss of two arginines (Δ331-344) by further truncation causes a loss of binding of Aurora B to kinetochores and to the midbody in the presence of native Aurora B. However, after ablation of the endogenous protein, this mutant localizes correctly and restores function. These results suggest strongly that this near C-terminal sequence plays a role in correct localization and function and contributes to the affinity of Aurora B for its binding sites.

Further truncation to amino acid 326 leads to loss of recruitment of Aurora B either in the presence or absence of endogenous protein. This mutant additionally does not restore Aurora B function. We have eliminated the possibility that these truncation mutants are massively denatured and do not localize for this reason. The mutants retain native kinase activity toward the well recognized Aurora B substrate histone H3, and these results contrast strongly with the suppression of phosphorylation of histone H3 upon rescue of Aurora B ablation with a dead kinase mutant. Furthermore, structural modeling suggests the truncations

performed are in an unstructured tail domain of Aurora B that should not contribute to the overall protein structure.

These results thus illustrate that the sequence 326–333 is essential for localization and function of Aurora B. The two arginines (332, 333) seem important for binding affinity at the kinetochore and the midbody. Our rescue results indicate that the sequence content in this near C-terminal domain, aside from a potential role for the two arginines, is not of consequence for its localization, as a complete replacement of (Δ313-344) by the C terminus of Aurora A restores both localization and function in cells depleted of endogenous Aurora B, a result parallel to that with the Δ331 mutant. Our results thus establish that the latent capacity exists in Aurora A C-terminal sequence to restore Aurora B truncation mutants to normal localization and function. It will be of substantial interest, in this context, to perform similar ablation and mutant rescue experiments with Aurora A to define its localization requirements in the context of sequence divergence from Aurora B.

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